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Patentanmeldung Nr. Patent application No. Demande de brevet n°

96308362.1

PRIORITY DOCUMENT

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Im Auftrag

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Proteins

Technical Field of the Invention

The invention relates to anti-freeze polypeptides (AFP's) food product containing AFP's.

Background to the Invention

Anti-freeze polypeptides (AFP's) have been suggested for improving the freezing tolerance of foodstuffs.

WO 90/13571 discloses antifreeze polypeptides produced chemically or by recombinant DNA techniques from plants. The AFP's can suitably be used in food-products such as ice-cream. Example 3B shows modified ice-crystal shapes if a water-ice mixture is frozen into a film in combination with 0.01 wt% of AFP.

WO 92/22581 discloses AFP's from plants which can be used for controlling ice crystal shape in ice-cream. This document also describes a process for extracting a polypeptide composition from intercellular spaces of plants by infiltrating leaves with an extraction medium without rupturing the plant cells.

WO 94/03617 discloses the production of AFP's from yeast and their possible use in ice-cream. WO 96/11586 describes fish AFP's produced by microbes.

Up till now, however the use of AFP's has not been applied to commercially available consumer products. One reason for this are the high costs and complicated process for obtaining AFP's. Another problem is that sources of the AFP's are either difficult to obtain in sufficient quantities (e.g. fish containing AFP's) or are not directly suitable for use in food products.

The present invention aims to provide novel antifreeze polypeptides which have the advantage that they can easily be obtained from an abundant natural source and which provide good properties to products in which they are used.

It has been found that antifreeze polypeptides which possess good recrystallisation inhibition properties can be obtained from carrots. In particular it has been found that antifreeze polypeptides obtained from carrots show markedly better properties as compared to polypeptides isolated from other root vegetables. In particular the antifreeze polypeptides of the invention are capable of providing good recrystallisation inhibition properties without significantly changing the crystal shape of the ice-crystals, therewith possible leading to more favourable properties e.g. soft ice-cream.

Applicants have found that the effective antifreeze polypeptides from carrots are generally characterised by an apparent Molecular Weight on SDS-PAGE of 38 kDa. Accordingly in a first aspect the invention relates to antifreeze polypeptides which can be obtained from carrots and which have an apparent molecular weight on SDS-PAGE of 38 kDa.

In this context it will be clear to the skilled person that due to variation e.g. in SDS PAGE, the apparent molecular weight can only be determined with some variation in the results. For the purpose of the invention these variations e.g. from 36 to 40 kDa are also embraced within the scope of the term "apparent Molecular Weight of 38 kDa".

Applicants also have found that the effective anti-freeze polypeptides according to the invention comprise fragments having an amino acid sequence as represented in the examples.

Accordingly in a second aspect the invention relates to polypeptides comprising fragments (A-E) having an amino acid sequence as follows:

(A) LEU-PRO-ASN-LEU-PHE-GLY-LYS

(B) ILE-PRO-GLU-GLU-ILE-SER-ALA-LEU-LYS

(C) LEU-THR-X-LEU-ASP-LEU-SER-PHE-ASN-LYS

(D) SER-LEU-ARG-LEU-SER-SER-THR-SER-LEU-SER-GLY-PRO-VAL-PRO-LEU-PHE-PHE-PRO-GLN-LEU-X-LYS

(E) X-X-GLU-VAL-ILE-PRO-X-GLN-LEU-SER-THR-LEU-PRO-ASN-LEU-LYS

Also embraced within the invention are alleles and derivatives of this polypeptide which still possess the antifreeze properties. Preferable the derivatives show at least 75% homology with the polypeptide of figure 1, more preferred more than 85%, most preferred more than 95%. For the purpose of the invention the term derivative also embraces modified polypeptides which still possess the antifreeze properties, for example glycosylated forms of the above polypeptides.

Although the proteins of the invention can easily be isolated from carrots, also genetic manipulation techniques may be used to produce the proteins described in the invention. An appropriate host cell or organism would be transformed by a gene construct that encodes the desired polypeptide. The nucleotide sequence coding for the polypeptide can be inserted into a suitable expression vector containing the necessary elements for transcription and translation and in a manner that they will be expressed under appropriate conditions (eg in proper orientation and

correct reading frame and with appropriate targeting and expression sequences). The methods required to construct these expression vectors are well known to those skilled in the art.

A number of expression systems may be utilised to express the polypeptide coding sequence. These include, but are not limited to, bacteria, yeast, insect cell systems, plant cell culture systems and plants all transformed with the appropriate expression vectors. Yeast, plants and plant culture systems are preferred in this context.

A wide variety of plants and plant cell systems can be transformed with the nucleic acid constructs of the polypeptides. Preferred embodiments would include, but are not limited to, maize, tomato, tobacco, carrots, strawberries, rape seed and sugar beet.

As described above vectors containing a nucleic acid sequence capable of encoding the polypeptides of the invention are also embraced within the scope of the invention. The invention also relates to nucleic acids capable of encoding the polypeptides of the invention, antibodies which specifically bind an (epitope of the) polypeptides. Also embraces are polypeptides which are immunologically related to the polypeptides as determined by its cross reactivity with an antibody raised against the above polypeptides.

Based on the above information it is also possible to genetically modify other natural sources such that they produce the advantageous AFP's as identified here-above.

Preferably those AFP's are chosen which have significant ice-recrystallisation inhibition properties. A suitable test for determining the recrystallisation inhibition

properties is indicated in the examples. Preferably AFP's in accordance to the invention provide a ice particle size (mean crystal length) upon recrystallisation of less than 50 μM, more preferred from 5 to 40 μm.

The AFP's can conveniently be used in several products, preferably in food products which are frozen or intended to be frozen.

Examples of such food products are: frozen food products such as vegetables, sauces, soups, snacks, frozen confectionery such as ice-cream or water-ice, dairy products etc.

The preferred products wherein the AFP's are used are or frozen vegetables or frozen confectionery products such as ice-cream or water-ice. Preferably the level of AFP's is from 0.0001 to 0.5 wt% based on the final product. If dry-mixes or concentrates are used, the concentration may be higher in order to ensure that the level in the final frozen product is within the above ranges. Surprisingly it has been found that compositions of the invention can contain very low amounts of AFP's while still being of good quality.

Up till now the general belief has been that fairly high levels of AFP's are required to obtain a reasonable improvement of recrystallisation inhibition properties. Surprisingly it has now also been found that for frozen products improved recrystallisation properties and increased temperature tolerance can already be obtained if low levels of AFP's are used. Preferred levels of AFP are from 0.01 to 0.3 wt%, more preferred 0.05 to 0.2 wt%.

For the purpose of the invention the term frozen confectionery product includes milk containing frozen confections such as ice-cream, frozen yoghurt, sherbet,

sorbet, ice milk and frozen custard, water-ices, granites and frozen fruit purees.

Preferably a the level of solids in the frozen confection (e.g. sugar, fat, flavouring etc) is more than 3 wt%, more preferred from 10 to 70wt, for example 40 to 70 wt%.

Frozen confectionery products according to the invention can be produced by any method suitable for the production of frozen confectionery. Especially preferably however all the ingredients of the formulation are fully mixed before the freezing process starts.

EXAMPLES

Example I

Carrots (Daucus caroto cv Autumn King) were grown in ndividual pots. When plants were approximately twelve weeks old, they were transferred to a cold room and held at 4°C in constant light during 4 weeks for cold-acclimation. Plants were watered three times a week.

Fresh tissue of the carrots were ground with a pestle and mortar (cooled to 4°C) in an equal volume buffer A (10mM EDTA, 20 mM Ascorbic acid, buffered with Tris to pH 7.4) held on ice. The homogenates were filtered through one layer of muslin and kept on ice prior to further use.

As a comparison several other root-plants were grown and homogenates prepared from the roots as above.

Anti-freeze activity was measured using a modified "splat assay" (Knight et al, 1988). 2.5 µl of the solution under investigation in 30% (w/w) sucrose was transferred onto a clean, appropriately labelled, 16 mm circular coverslip. A second coverslip was placed on top of the drop of solution and the sandwich pressed together between finger and thumb.

The sandwich was dropped into a bath of hexane held at -80°C in a box of dry ice. When all sandwiches had been prepared, sandwiches were transferred from the -80°C hexane bath to the viewing chamber containing hexane held at -6°C using forceps pre-cooled in the dry ice. Upon transfer to -6°C, sandwiches could be seen to change from a transparent to an opaque appearance. Images were recorded by video camera and grabbed into an image analysis system (LUCIA, Nikon) using a 20x objective. Images of each splat were recorded at time = 0 and again after 30-60 minutes. The size of the ice-crystals in both assays was compared. If the size at 30-60 minutes is similar or only moderately increased compared to the size at t=0, this is an indication of good ice-crystal recrystallisation properties.

Results: from the sandwich splat assay test it appeared that samples from carrot roots, carrot stem and carrot leaves possess significant ice-recrystallisation inhibition properties, whereby the roots and leaves are most active. As a comparison a sample of non-acclimated carrot roots was tested, which showed significant less activity. For the following examples root tissue was used for further testing on carrots.

As a comparison several other vegetable roots were investigated by means of the sandwich splat assay test in 30% sucrose. Among these vegetables were turnip, kale, brussels sprout, wintergreen cabbage, rape, pak choi, parsnip and strawberry. None of these sources of material provided significant ice-recrystallisation inhibition activity.

Example II

Carrot root tissue was homogenized in three volumes (w/v) buffer (20mM ascorbic acid, 10 mM EDTA, 50 mM Tris/HCL, pH

7.2) in a pre-cooled pestle and mortar and filtered through one layer of muslin. The filtrate was centrifuged at 6,000g, ten minutes at 4°C; the supernatant was collected and centrifuged at 100,000g for 1 hour at 4°C. The 100,000g supernatant from this step is termed the soluble fraction and the pellet the microsomal fraction.

The supernatant was applied to a 30 ml fast flow Q Sepharose (Pharmacia) column pre-equilibrated in 50 mM Tris/HCL pH 7.4 at a flow rate of 5 ml/min supplied by a HiLoad pump P-50 controlled by a Gradifrac low pressure chromatography system (Pharmacia) at 4°C and the eluate monitored at OD 280 by a UV monitor (Monitor UV1, Pharmacia) recorded on a chart recorder (REC 102, Pharmacia). 5 ml fractions were collected. The column was washed with 50mM Tris/HCL pH 7.4 at the same flow rate until the OD 280 returned to zero. A 150ml gradient of 0-0.4 M NaCl in Tris/HCL pH 7.4 was then applied followed by a 2 M NaCl column wash. Eluate fractions were subjected to the splat assay as in example I.

Fractions containing anti-freeze activity were pooled and concentrated using polyethylene glycol as follows: the fractions were transferred in 10kDa cut off dialysis tubing (Sigma) which had been washed in tap water, boiled in 50mM EDTA pH 7.5 for 10 minutes and rinsed in milli Q water. The dialysis tubing containing the sample to be concentrated was covered with solid polyethylene glycol compound Mol. Wt. 15,000 - 20,000 (Sigma) and incubated at 4°C for up to 4 hours or until the sample volume inside the dialysis tubing had reduced up to 10 fold.

The pooled concentrate from the Q sepharose column was applied either to a phenyl Sepharose column, a SMART superdex 75 gel permeation column or an FPLC superdex 75 gel permeation column.

Carrot root anti-freeze proteins were purified by gel permeation chromatography as follows:

20µl aliquots of sample were applied to a SMART superdex 75 column (Pharmacia) pre-equilibrated in 50mM Tris/HCl pH7.4 containing 0.15M NaCl (Buffer E) at a flow rate of 40µl/min and components separated by gel permeation at the same flow rate in equilibration buffer. The eluate was monitored at OD 280 and OD 215. 80µl fractions were collected between 0.85 and 0.89ml, 40µl fractions between 0.89 and 1.24ml and 100µl fractions between 1.24 and 3.0 ml. The void volume (Vo) of the column was 0.91 ml as determined by the retention volume of a solution of Blue Dextran. The superdex column was calibrated by application of 10µl of a solution containing 5mg/ml BSA (Mr 66kDa, retention (Ve)=1.02 ml), 3mg/ml Carbonic anhydrase (Mr 29 kDa, Ve=1.22 ml), 2mg/ml Cytochrome C (Mr 12.4 kDa, Ve=1.41 ml) and 2mg/ml Aprotinin (Mr 6.5 kDa, Ve=1.59 ml) and a standard curve plotted of Ve/Vo against log Mr. Fractions containing anti-freeze activity were identified by the spat assays in Example I, with an activity peak that showed a retention volume of 1.16 ml and an apparent molecular weight of 40 kDa. These measurement confirmed that the 38 kDa band from cold acclimatised carrots was an anti-freeze peptide.

SDS-PAGE was carried out according to Laemmli (1970) using the Biorad mini system. Samples to be analyzed by SDS-PAGE were dissolved in SDS-PAGE sample buffer (Laemmli 1970), heated for 5 minutes at 100°C on a dry heating block (Techne) and centrifuged for 3 minutes at 10,000g at room temperature. Samples (10-50µl) were applied to mini-gels (Biorad, 0.75,1.0 or 1.5mm thickness, 10,12,15% acrylamide or 10-20% gradient acrylamide (pre-poured from Biorad)) and electrophoretically separated. Separated polypeptides were fixed and stained in the gel either with Coomassie blue (0.1% (w/v) Coomassie Brilliant Blue in acetic

acid/methanol/miliQ water (5:4:31, by vol)) or silver stained using the Biorad silver stain kit according to the manufacturer's instructions. Gels were dried between two sheets of Gelair cellophane in a Biorad gelair dryer according to the manufacturer's instructions. Sigma high and low range molecular weight marker kits were used according to the manufacturer's instructions for determination of apparent M_r on SDS-PAGE.

The ion exchange chromatography was carried out with cold acclimatised carrot root and non-cold acclimatised carrot root. The resulting gel SDS-PAGE gels showed the presence of a 38kDa band in the cold acclimatised sample. This band was much less abundant in the non-cold acclimatised root. This 38kDa band was hence attributed to anti-freeze activity.

Example III

For protein sequencing, the 38kDa carrot root protein was purified as described in the previous example and then to ensure further purification the sample to be sequenced was excised from the SDS PAGE gel and then proteolytically digested *in situ* in the polyacrylamide gel slice.

Preparations of largely pure 38 kDa protein, that still had some minor contaminating proteins, were loaded onto a 12% polyacrylamide gel. Three lanes each with 2 µg of protein were loaded and electrophoresed in the gel until the dye front reached the bottom of the gel. The gel was then stained in 0.2% coomassie brilliant blue (w/v), 30% methanol (v/v), 1% acetic acid (v/v) for 20 minutes and then detain with 30% methanol until the protein bands could be visualised. The 38 kDa band was identified by comparison with molecular weight markers loaded into adjacent lanes and the band from each lane was excised with a scalpel blade, taking care to exclude contaminating bands.

The gel slices were transferred to a clean eppendorf tube and washed twice with 0.5ml of 50% acetonitrile (v/v), 100mM Tris/Cl, pH 8.5. The washing removed some of the coomassie stained and also partially dehydrated the gel slices. The gel slices were then removed from the tube and subjected to air drying on the laboratory bench until they had shrunk significantly and started to curl up. They were then transferred back to the eppendorf and rehydrated with firstly, 10µl of 100mM Tris/Cl, pH 8.5 containing 1µg of endoproteinase Lys C (Boehringer Mannheim). This is a proteinase that specifically cleaves polypeptide chains on the carboxy terminal side of lysine residues. Further Tris buffer was added to the gel slices until they were fully rehydrated and they were then incubated at 37°C for 16 hours.

After incubation 1µl of trifluoroacetic acid was added to the tube to stop the reaction and then the gel slices were washed twice with 0.3ml of 60% acetonitrile (v/v), 0.1% TFA (v/v) at 30°C for 30 minutes. This was to again partially dehydrate the gel slices causing them to shrink and elute the peptides that had been generated. The supernatant was transferred to another clean eppendorf tube and then dried in a centrifugal evaporator for 2 hours until the sample was near dryness and resuspended to a volume of 0.1ml with 0.1% TFA.

The peptides were then separated by reversed phase HPLC on a Smart micropurification system (Pharmacia). The peptide digest was loaded onto a C18 column (2.1 x 100 mm) equilibrated in 0.1% TFA (Solvent A) at a flow rate of 0.1ml min. The column was then eluted with a gradient of 0 - 70% of Solvent B (90% acetonitrile v/v, 0.085% TFA v/v) over 70 minutes at the same flow rate. The optical density was monitored at 214 nm and individual peptide peaks were collected in the fraction collector by manual stepping. Polypeptides were sequenced by loading onto a model 492 Perkin Elmer protein sequencer using the liquid phase chemistry cycles as recommended by the manufacturer.

Several polypeptide fragments (A-E) were analyzed in the 38 kDa band and had sequences substantially homologous to:

(A) LEU-PRO-ASN-LEU-PHE-GLY-LYS

(B) ILE-PRO-GLU-GLU-ILE-SER-ALA-LEU-LYS

(C) LEU-THR-X-LEU-ASP-LEU-SER-PHE-ASN-LYS

(D) SER-LEU-ARG-LEU-SER-SER-THR-SER-LEU-SER-GLY-PRO-VAL-PRO-LEU-PHE-PHE-PRO-GLN-LEU-X-LYS

(E) X-X-GLU-VAL-ILE-PRO-X-GLN-LEU-SER-THR-LEU-PRO-ASN-LEU-LYS

Example IV

Cell cultures for producing anti-freeze proteins were made as follows:

New cell cultures were initiated based on methods described in Gamborg and Wetter 1975, Torres 1989, Dodds and Roberts 1985.

Cold acclimatised Carrot (Autumn King): the surface of the storage root was sterilized firstly by washing with 10% Teepol detergent, followed by scrubbing under running water then rinsing under running water for 15 minutes. Where practicable (on the basis of size) the root was peeled. The root was then aseptically cut into 0.5cm slices, which were placed in 70% v/v ethanol for 10 minutes with shaking followed by 10% v/v Domestos + 2 drops Tween 20 (Sigma) for 25 minutes, also with shaking. Sections were then washed 3 x with sterile distilled water. Cylinders of approximately 0.5cm diameter were cut through the slices using a sterile scalpel, and the remainder cut into 2-3mm lengths. These tissue discs (explants) were aseptically transferred onto solid MS medium containing 30g/l sucrose, 10mg/l indole acetic acid (IAA), 0.1 mg/l kinetin and 8g/l technical agar, which was contained in 60ml Sterilin containers. The explants were incubated in the dark at 20°c.

When necessary, the resulting calli were divided into smaller sections, which were plated onto fresh medium. Suspension cultures were then initiated from actively growing callus.

Additionally carrot cell suspension culture lines (NOR and OX6) were obtained from the Department of Biochemistry and Molecular Biology, University of Leeds. 10 ml of these cultures were sub-cultured into 90ml of fresh Murashige and Skoog medium (Sigma) containing 25g/l sucrose and 1mg/l

2,4-D every seven days. Cultures were incubated in an orbital shaking incubator at 150 rpm at 25°C in the dark.

The NOR culture was cold treated as follows:

18x5ml 7d old NOR culture was added to 18 x 100ml Erlenmeyer flasks containing 45ml carrot MS medium. The cultures were incubated at 25°C as previously described for 4 days, then the incubator temperature reduced to 4°C. Two flasks were removed immediately and the cells and medium harvested as previously described as t = 0. The remaining flasks were harvested in duplicate at t=8h,1d,2d,4d,7d,9d,11d, and 14d.

The cold acclimation treatment was repeated using larger cultures of both NOR and OX6, which were transferred to 4°C after 4d and 7d of growth at 25°C. Cultures were harvested at t=0, t=7d and t=14d. In addition to harvesting, the PCV was determined for each culture at each time point.

The NOR cold acclimated cells were prepared for splat analysis as in Example I as follows: Quick frozen cells were ground to a fine powder in liquid nitrogen using a pestle and mortar. The powdered samples were resuspended in 2 x volume of 10mM EDTA + 20mM ascorbic acid, whirlimixed for 30 seconds then centrifuged at 10.000g for 10 minutes. 10µl aliquots of the supernatants were splatted using the buffer control as a negative control. RI activity could be detected in cold acclimated cells and medium but not in the non-cold acclimated samples.

The media samples from NOR suspension were analyzed as follows. The NOR carrot medium was buffered by addition of 100µl of 1M Tris/HCl pH 7.4. This was then applied to the 1 ml Q Sepharose column (Pharmacia) at a flow rate of 1

ml/min and bound molecules eluted with 3 ml aliquots of 50 mM Tris/HCl pH 7.4 containing concentrations of 0.5 M NaCl. 1 ml fractions were collected.

This anion exchange method was also used to fractionate t=0,2d,4d,7d,11d, cold acclimated and t=7d non-cold acclimated medium samples. Fractions were tested for activity by sandwich splat assay as described in example I.

The antifreeze activity in culture medium was purified by gel permeation chromatography as follows.
The 14 cold acclimated 0.5M NaCl eluate from the Q sepharose column (fraction 2) from above was acetone precipitated and the pellet resuspended in 50µl 50mM Tris/HCl + 0.15 M NaCl, pH 7.2. This was then centrifuged at 10.000g for 10 minutes, and 20µl loaded onto a Superdex 75 gel permeation column on the Pharmacia SMART system. The flow rate was 40µl/min and the mobile phase was 50mM Tris/HCl + 0.15M NaCl, pH7.2. 80µl fractions were collected and splatted. This procedure was repeated using t=14d non-cold acclimated 0.5M NaCl eluate from the Q sepharose column and fresh medium.

Further isolation of the active proteins can be done by SDS PAGE analysis in line with example II.

Example V preparation of ice-cream

Root extract from cold acclimatised carrot roots was prepared by scrubbing freshly pulled cold acclimatised (as in example I) carrots in cold water. The tops are removed and the juice extracted employing a domestic juice extractor (Russell Hobbs, model no 9915). The juice was frozen in 1 litre blocks and stored a -20°C prior to collection for use in ice cream trials.

The carrot AFP juice was added to the following ice cream formulation

INGREDIENT	parts by weight
Skimmed Milk Powder	10.000
Sucrose	13.000
MD40	4.000
Locust Bean Gum	0.144
Genulacta L100	0.016
MGP	0.300
Butteroil	8.000
Vanillin	0.012
Water	64.528
Carrot Extract (from cold acclimated carrots containing 1-10 mg AFP per kg)	4.472

Ice-cream was prepared by freezing above formulation and aeration to 106% overrun.

Measurements were made on fresh sample and on samples which had been abused by storage at -10 °C for a period of 10 days. As a comparison a sample without carrot extract was measured in the same way. The measurements were done as follows:

Samples were equilibrated at -18 C in a Prolan Environmental cabinet for approximately 12 hours. Three samples were chosen representatively from each batch of ice cream and a slide was prepared from each in a Cryostat temperature control cabinet by smearing a thin layer of ice cream from the centre of each block onto a microscopic slide. A single drop of white spirit was applied to the slide and a cover slip was then applied. Each slide, in turn, was then transferred to a temperature controlled

microscope stage (Leit LaborLux S, Leica x10 objective, temperature -18 °C). Images of ice-crystals (about 400 individual ice-crystals) were collected and relayed through a video camera (Sanyo CCD) to an image storage and analysis system (LEICA Q520MC).

The stored ice crystal images were highlighted manually by drawing around the perimeter which then highlights the whole crystal. Images of the highlighted crystals were then measured using the image analysis software which counts the number of pixels required to complete the longest straight line (length), shortest straight line (breadth), the aspect ratio (length/breadth). The data for each individual ice crystal of a batch of ice cream was imported into a spreadsheet where analysis of the data set was carried out to find the mean, and standard deviation.

The ice Cream Hardness Measurements were carried out using a Hounsfield H10KM Universal Tester, a Hounsfield 100N Load Cell and a 10cm Cylindrical Stainless steel probe. The ice-cream samples were prepared by 16 Hour incubation of 486ml ice cream blocks in a Prolan Temperature Control Cabinet set at -18 °C.

The ice cream block was removed from Prolan temperature control cabinet and placed the Hounsfield H10KM Universal Tester. The 10cm cylindrical probe was pushed into the ice cream block at a constant rate of 400mm/min to a depth of 20mm. The maximum force recorded during the compression was used and expressed as the ice cream Hardness. If cracking or brittle fracture of the sample was observed this was indicated in the right hand column

The following results were obtained

	Ice Crystal Size Parameters				Material Properties	
Sample	Mean Crystal Length / um	Mean Crystal Breadth / um	Mean Crystal Shape Factor / -	Mean Crystal Aspect Ratio / -	Hardness / N	Brittle Fracture observation
Carrot AFP - fresh	26.79 ± 1.3	19.00 ± 0.9	1.15 ± 0.013	1.43 ± 0.024	40.8	Yes
Carrot AFP - Abused	33.48 ± 1.3	24.61 ± 0.9	1.13 ± 0.013	1.37 ± 0.020	59.9	Yes
Cont.- Fresh	33.67 ± 1.1	24.79 ± 0.8	1.12 ± 0.008	1.38 ± 0.018	27.3	No
Cont.- Abused	61.77 ± 2.7	46.54 ± 2.0	1.11 ± 0.010	1.37 ± 0.020	32.7	No

The following conclusions can be drawn:

a) Initial ice crystal size is smaller in ice cream containing Carrot AFP, thus carrot AFP is inhibiting recrystallization inhibition.

b) Ice crystals in carrot AFP ice cream are retarded in their recrystallization processes.

c) Ice crystal shape in carrot AFP ice creams are not significantly different from crystal shapes seen in conventional ice creams.

d) Material properties of ice cream containing carrot AFP are modified from those noted for conventional ice cream. Namely, ice creams are harder than conventional ice cream but still softer than ice-cream containing e.g. fish AFP's. Secondly, ice cream containing carrot AFP was observed to fracture.

CLAIMS

1. Antifreeze polypeptides which can be obtained from carrots and which have an apparent molecular weight on SDS-PAGE of 38 kDa and alleles or derivatives thereof.

2. Antifreeze polypeptides comprising fragments (A-E) having an amino acid sequence as follows:

(A) LEU-PRO-ASN-LEU-PHE-GLY-LYS

(B) ILE-PRO-GLU-GLU-ILE-SER-ALA-LEU-LYS

(C) LEU-THR-X-LEU-ASP-LEU-SER-PHE-ASN-LYS

(D) SER-LEU-ARG-LEU-SER-SER-THR-SER-LEU-SER-GLY-PRO-VAL-PRO-LEU-PHE-PHE-PRO-GLN-LEU-X-LYS

(E) X-X-GLU-VAL-ILE-PRO-X-GLN-LEU-SER-THR-LEU-PRO-ASN-LEU-LYS

and alleles or derivatives thereof

3. Method of obtaining polypeptides according to one or more of claims 1-2 whereby the polypeptide is isolated from cold-acclimatised carrots.

4. Method of obtaining polypeptides according to one or more of claims 1-2, whereby the polypeptide is expressed by a genetically modified organism preferably selected from the group of microorganisnm, plants or plant cell cultures.

5. Microorganism capable of expressing the polypeptide of claim 1 or 2.

6. Vector being capable of being inserted into an

organism such that this organism produces the polypeptide of claim 1 or 2.

7. Cell culture capable of producing the polypeptide of claim 1 or 2.

8. A nucleic acid capable of encoding the polypeptide of claim 1 or 2.

9 An antibody capable of specifically binding the polypeptide of claim 1 or 2.

10. A polypeptide that is immunologically related to the polypeptide of claim 1 or 2 as determined by its cross reactivity with an antibody of claim 9.

11. Food product (with the exception of carrots) comprising a polypeptide of claim 1, 2 or 10.

12. Food product of claim 11 being a frozen confectionery product or a frozen vegetable.

13. Use of the polypeptides of claim 1 or 2 for increasing the frost tolerance of plants.

Abstract

Novel antifreeze polypeptides can be isolated from carrots. These peptides can favourable influence the properties of consumer products e.g. frozen confectionery products.